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Winter soil freeze-thaw cycles lead to reductions in soil microbial biomass and activity not compensated for by soil warming

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ABSTRACT

Air temperatures are rising and the winter snowpack is getting thinner in many high-latitude and high-elevation ecosystems around the globe. Past studies show that soil warming accelerates microbial metabolism and stimulates soil carbon (C) and nitrogen (N) cycling. Conversely, winter snow removal to simulate loss of snow cover leads to increased soil freezing and reductions in soil microbial biomass, exoenzyme activity, and N cycling. The Climate Change Across Seasons Experiment (CCASE), located at Hubbard Brook Experimental Forest, NH (USA) is designed to evaluate the combined effects of growing season soil warming and an increased frequency of winter soil freeze-thaw cycles on a northern forest ecosystem. Soils were collected from CCASE over two years (2014 and 2015) and extractable C and N pool sizes, as well as microbial biomass, exoenzymes, and potential net N mineralization and microbial respiration were measured. Soil warming alone did not stimulate microbial activity at any sampling time. Extractable amino acid N and organic C, proteolytic and acid phosphatase activity, and microbial respiration were reduced by the combination of warming in the growing season and winter soil freeze-thaw cycles during the period following snowmelt through tree leaf out in spring. The declines in microbial activity also coincided with an 85% decline in microbial biomass N at that time. Growing season warming and winter soil freeze-thaw cycles also resulted in a two-fold reduction in phenol oxidase activity and a 20% reduction in peroxidase activity and these declines persisted throughout the snow-free time of the year. The results from this study suggest that positive feedbacks between warming and rates of soil C and N cycling over the next 100 years will be partially mitigated by an increased frequency of winter soil freeze-thaw cycles, which decrease microbial biomass and rates of soil microbial activity.

1. Introduction

Understanding the consequences of climate warming on soil microbial activity is important due to the potential feedbacks to global air temperature rise (Cox et al., 2000; Davidson and Janssens, 2006; Friedlingstein et al., 2006; Melillo et al., 2011). In recent decades, rising air temperatures have reduced the depth and duration of winter snow in temperate ecosystems that have historically experienced a seasonal snowpack (Hamburg et al., 2013; Henry, 2008; Kreyling and Henry, 2011). Winter snow cover plays a critical role in mediating soil nutrient cycling because the physical characteristics of snow, such as high surface albedo and low thermal conductivity, produce an insulating effect that maintains increased soil temperature relative to air temperature during winter (Zhang, 2005). In the northeastern U.S., mean annual air temperatures have risen by approximately 1 °C over the last 50 years (Hamburg et al., 2013), with air temperatures in winter rising at a faster rate (+0.7 °C decade^{-1}) compared to summer (+0.1 °C decade^{-1}; Hayhoe et al., 2007). Increasing winter air temperature over the next 100 years will reduce the depth and duration of winter snow cover, likely causing colder soil temperatures and more frequent soil freeze-thaw cycles in many temperate forest ecosystems (Brown and DeGaetano, 2011; Campbell et al., 2010).

Winter snow and soil temperature conditions are increasingly recognized as critical factors that mediate soil carbon (C) and nitrogen (N) pool sizes and process rates, both overwinter and during the transition between winter and spring (Brooks and Williams, 1999; Giblin et al., 1991; Groffman et al., 2006; Reinmann et al., 2012). Winter snow cover can prevent soils from freezing and thus allows microbial biomass production, exoenzyme synthesis, and nutrient immobilization to be higher compared to when snow is absent and soils freeze (Brooks and

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Williams, 1999; Drake et al., 2013; Kuhnert et al., 2012; Ueda et al., 2013). By contrast, when the winter snowpack is thin or intermittent, soil freezing limits substrate diffusion, increases microbial mortality and reduces microbial population size, and also reduces exoenzyme production following snowmelt in spring (Boutin and Robitaille, 1995; Deluca et al., 1992; Fitzhugh et al., 2001; Matzner and Borken, 2008; Skogland et al., 1988; Sorensen et al., 2016). Thus, an increase in the frequency of soil freeze-thaw cycles due to rising winter air temperature may be associated with reductions in microbial biomass, exoenzyme production, and soil C and N cycle process rates, especially in the spring time following winter snowmelt.

Warmer soil temperatures during snow-free times of the year are known to increase the activity of microbial exoenzymes (Brzostek and Finzi, 2011; Davidson and Janssens, 2006; Schindlbacher et al., 2015), increase the pool sizes of dissolved C and N (Bai et al., 2013; Lu et al., 2013), and are associated with increased rates of net N mineralization and soil respiration in temperate forest ecosystems (Fahey et al., 2005; Giasson et al., 2013; Rustad et al., 2001). Soil warming in temperate forest ecosystems is typically applied with the aim of simulating a projected 2–5 °C increase in mean annual air temperature by the year 2100 (Bai et al., 2013; Melillo et al., 2002). In contrast to soil warming manipulations, other researchers have reduced the winter snowpack to examine the effects of warmer winter air temperatures and increased soil freezing severity on temperate forests (Aanderud et al., 2013; Groffman et al., 2001). Soil freeze-thaw cycles increase dissolved C and N in soil solution in the following growing season (Fitzhugh et al., 2001), which is similar to the effects of soil warming. But in contrast to warming, reductions in winter snow coverage reduce rates of exoenzyme production, net N mineralization, and nitrification in temperate forest ecosystems (Durán et al., 2014; Sorensen et al., 2016). Thus, an increased frequency of winter freeze-thaw cycles may partially offset the stimulatory effect of warmer soils on soil microbial metabolism. Yet effects of winter soil freeze-thaw cycles combined with soil warming during snow-free periods have to date been generally untested. This critical gap in knowledge precludes a comprehensive understanding of the response of soil C and N cycle processes in temperate forests to global air temperature rise.

The purpose of the Climate Change Across Seasons Experiment (CCASE), located at Hubbard Brook Experimental Forest (HBEF), New Hampshire, USA is to evaluate the combined effects of elevated soil temperatures in the growing season and an increased frequency of winter soil freeze-thaw cycles in a northern forest ecosystem (Templer et al., 2017). We collected soils in the first two years (2014 and 2015) following initiation of treatments at the CCASE experiment. We tested two hypotheses: (1) soil warming increases potential exoenzyme activity, net N mineralization, and respiration rates during snow-free months of the year. In addition, we hypothesized that (2) winter soil freeze-thaw cycles would offset the stimulatory effect of soil warming on microbial biomass, exoenzyme activity, net N mineralization, and microbial respiration during spring only, but not during later snow-free periods of the year.

2. Materials and methods

2.1. Field site description

The HBEF is a part of the White Mountain National Forest, which is located in central New Hampshire, USA (43.56° N, 71.45° W). The elevation at HBEF ranges from approximately 225 m asl to approximately 1100 m asl. Sites used in this study are located at approximately 225 m asl and are dominated by red maple (*Acer rubrum*) in the canopy with an understory composed of mostly American beech (*Fagus grandifolia*). The soils at Hubbard Brook are acidic (pH 3.9) Typic Haplorthods with an organic layer consisting of leaf-litter (Oi), dense root-mat and decomposing organic material (Oe), and a nutrient-rich humus layer (Oa); all of which together extend to approximately 6.5 cm below the soil surface (Bohlen et al., 2001).

Historically, a snowpack begins to develop in December, reaches a maximum depth of 70–100 cm, and persists until April. In years with below-average snowfall, soil frost can last from December through April or May in this region (Fahey and Lang, 1975). Mean annual soil frost depth is 5.8 cm over the last 50 years, ranging annually from 0 cm to 25 cm below the soil surface (Campbell et al., 2010). Mean air temperature from December to March was −4 °C from 1955 to 2012, and ranges from an average minimum of −12 °C during January to an average maximum of 19 °C during July (Bailey et al., 2003; Bohlen et al., 2001). During the last half century, winter air temperatures have risen by 2.5 °C, the maximum depth of winter snowpack has declined by 26 cm, and the duration of winter snow cover has declined by four days per decade (Bailey et al., 2003; Burakowski et al., 2008; Hamburg et al., 2013).

2.2. Experimental design and climate change treatments

CCASE was established at HBEF in July 2012 and is ongoing (Templer et al., 2017). There are six 11 m x 13.5 m experimental plots, with each plot centered on at least three mature red maple trees and composed of a variety of other hardwood tree species. Two plots serve as reference controls. Four other plots receive one of two experimental treatments: soil warming during the snow-free periods of the year (hereafter referred to as “Warmed” plots; n = 2 plots) or soil warming during snow-free periods plus an increased frequency of winter soil freeze-thaw cycles (hereafter referred to as “Warmed + FTC” plots; n = 2 plots). Large-sized plots were chosen because the main objective of CCASE is to understand both above- and belowground responses to changes in climate. Further, the CCASE experiment is not fully factorial due to the high financial cost associated with establishing and engineering the initial infrastructure and the ongoing cost associated with soil warming. CCASE is similar to other experiments that have not employed a fully-factorial experimental design due to logistical constraints (e.g. Aerts et al., 2004); but rather chosen experimental treatments to most accurately simulate projected changes in climate for the northeastern U.S. region (Campbell et al., 2010).

Buried heating cables were used to achieve soil warming during the growing season in the Warmed and the Warmed + FTC treatment plots, which was intended to simulate soil warming that occurs as a result of an anticipated +5 °C rise in air temperature by the year 2100 (Hayhoe et al., 2007). The heating cables do not warm the air aboveground, thus the heating cables do not fully capture the tree response to warming air temperature and it is possible that we would have observed different plant-soil-microbial feedbacks had the warming been induced using warmed air. Further critical discussions about the advantages and artefacts associated with ecosystem warming methods can be found elsewhere (Aronson and McNulty, 2009; De Frenne, 2015; Henry, 2012; Sanders-DeMott and Templer, 2017).

Warming cables were installed in July and August 2012 in 56 parallel lines across each treatment plot (each 10 cm deep and separated by 20 cm) created using a thin drywall spatula and burying the cable within a thin cut in the soil. We created similar cuts in the Reference treatment, but no cables were installed. Soil temperatures in all six plots were measured throughout the year using thermistors (Betatherm type 10K3A1) buried at 10 cm (n = 6 per plot in heated plots and n = 4 per plot in reference plots) depth below the surface. Additional sensor infrastructure in each plot is described in Templer et al. (2017).

Snow was removed via shoveling from the Warmed + FTC treatment beginning in December 2013 within 48 h of snowfall to induce soil freezing. We left a 3–5 cm layer of packed snow at the soil surface to avoid compacting of the soil during subsequent snow- and shoveling events and to maintain surface albedo. We operationally defined soil freezing as soil temperatures remaining below −0.5 °C at 10 cm depth for 72 h. Following soil freezing, the heating cables were turned on in the Warmed + FTC treatment in order to warm the soils to +1 °C for

72 h, after which time the heating cables were turned off. The entire cycle of soil freezing to below −0.5 °C for 72 h and warming to +1 °C for 72 h defined a soil freeze-thaw cycle. We created four freeze-thaw cycles in both winters to mimic the four additional freeze-thaw cycles that are projected annually for this region of the northeastern U.S. over the next century (Campbell et al., 2010).

We initiated the soil warming treatments following snowmelt and at the start of the snow-free period of the year, which was operationally defined as the rapid increase in soil temperature due to loss of snow cover during snowmelt (Groffman et al., 2012). We initiated soil warming treatments in the Warmed and the Warmed + FTC treatment plots on April 18 and April 21 in 2014 and 2015, respectively. The heating cables were shut off at the end of the snow-free period, which we defined as either five consecutive days with mean daily air temperatures below 0 °C or December 1, whichever occurred first. These dates corresponded to November 19, 2014 and December 1, 2015. Experimental treatments were initiated in December 2013, allowing each plot 16 months to recover following cable installation before the treatments began. In order to assess potential differences in soil properties caused by cable installation, we took three 10 cm × 10 cm organic horizon soil samples per plot in July 2013 and measured soil bulk density, pH in a 1:2 w/vol slurry in MilliQ H_2O, and total soil N and C, and soil C:N ratio via flash combustion (Sorensen et al., 2016). See Templer et al. (2017) for further description of the experimental design and technical details of the experimental treatments.

2.3. Sample collection and processing

Snow depth in winter was measured weekly using meter sticks. Soil frost depth was measured using frost tubes consisting of tubing filled with methylene-blue dye and inserted into PVC casings that extended approximately 50 cm below the soil surface (Rickard and Brown, 1972).

Soils were collected on six dates in 2014 and four dates in 2015 to determine the microbial response to experimental treatments. In year 2014 soils were collected at snowmelt (April 18), tree bud break (Early May–May 12), tree leaf out (Mid-May - May 21), peak aboveground production (June 1 and July 20), and at the onset of autumn plant senescence (September 24, 2014). In year 2015 soils were collected at tree bud break (Early May–May 12), tree leaf out (Mid-May, May 21), and peak aboveground production (June 1 and July 23).

Four organic horizon soil samples were taken from each plot using a soil knife and a 10 cm × 10 cm frame to define the vertical edges of the sample at each sampling time. Organic layer depth ranged from 3 to 10 cm. The soils were collected in the middle and in between parallel heating cables, which were 20 cm apart, to avoid collecting soils directly in contact of and to avoid severing the heating cables. A total of 144 soils were collected in 2014 (6 plots x 6 sampling times x 4 samples per plot) and 96 soils collected in 2015 (6 plots x 4 sampling times x 4 samples per plot). Soils were transferred from the field in a cooler on ice and sieved to < 2 mm to remove roots and coarse woody debris within 24 h of field collection. A 10 g subsample was placed in a drying oven at 65 °C for 48 h to determine the gravimetric water content (hereafter referred to as “soil moisture”) within 48 h of sample collection. Volumetric soil moisture measured over an integrated 0–30 cm depth profile did not differ across treatments in 2014 or 2015 at CCASE (Templer et al., 2017).

2.4. Soil microbial biomass N, extractable organic C, and exoenzyme activity

Microbial biomass N was determined on each sampling date using the chloroform fumigation-extraction method (Brookes et al., 1985). Following alkaline persulfate digestion (Cabrera and Beare, 1993), total N in the fumigated and non-fumigated soils was determined by measuring nitrate (NO_3^-) colorimetrically (λ = 540 nm) using a Versamax microplate spectrophotometer (Molecular Devices, LLC; Sunnyvale, CA; Doane and Horwath, 2003). Microbial biomass N (μg N g dry soil^{-1}) was calculated as the difference in N concentration between the fumigated and non-fumigated soils, corrected for water content of the soil. We did not apply an extraction efficiency correction factor.

Table 1
Edaphic properties measured one year (summer 2013) following buried-heating cable installation, but prior to the establishment of experimental treatments. Treatment means and standard deviations (n = 2) were calculated from plot means of replicate cores (n = 3) taken within a single plot. Units for bulk density are (g cm^{-3}), and units for Total Soil C and Total Soil N are (g m^{-2}).

Response	Reference	Warmed	Warmed + FTC
Soil Bulk Density	0.36 ± 0.06	0.33 ± 0.04	0.36 ± 0.06
Soil pH	4.5 ± 0.1	4.5 ± 0.1	4.3 ± 0.1
Total Soil C	1987 ± 712	1255 ± 253	1252 ± 193
Total Soil N	73 ± 24	53 ± 9	53 ± 7
Soil C:N	25.6 ± 4.2	22.9 ± 4.6	23.3 ± 1.7

We used the same non-fumigated K_2SO_4 extractions that were used for estimating microbial biomass and the Mn(III)-pyrophosphate oxidation method (Bartlett and Ross, 1988), in order to estimate extractable dissolved organic C pool sizes. We applied correction factors empirically-derived for our field site in order to quantify extractable organic C pool sizes (Giasson et al., 2014).

The potential activity of four hydrolytic microbial exoenzymes—acid phosphatase (AP), β-N-acetylglucosaminidase (NAG), β-1,4-glucosidase (BG) and cellobiohydrolase (CBH)—were measured in each soil core on each sampling date in both 2014 and 2015. AP mineralizes phosphate groups from soil organic matter, NAG hydrolyzes amino sugars and chitin in soil, and both BG and CBH decompose components of cellulose. We determined the activity-saturating concentration (AP – 4000 μM; NAG – 2000 μM; BG – 2000 μM; CBH – 1750 μM) of methylumbelliferone (MUB) -linked substrate by incubating a subset of soils across a range of substrate concentrations. A soil slurry consisting of 1.5 g field-moist soil in 100 mL 250 mM sodium acetate buffer was mixed on a stir plate for 1 min. Eight 200 μL analytical replicates were aliquoted onto black 96-well flat-bottom microplates along with 50 μL MUB-linked substrate. The microplates were incubated for 2 h at 20 °C before enzyme activity was determined fluorometrically (Gemini XS, Molecular Devices; Sunnyvale, CA).

Phenol oxidase and peroxidase, two oxidative enzymes associated with the oxidative breakdown of soil organic C, were also measured on each sampling date. A soil slurry of 1.5 g field-moist soil in 100 mL 50 mM sodium acetate buffer was mixed on a stir plate for 1 min. Eight 200 μL analytical replicates were aliquoted onto 96-well clear, flat-bottom microplates along with 50 μL 25 mM L-DOPA. 20 μL of 0.3% hydrogen peroxide solution was added for the peroxidase assays. Enzyme activity was determined colorimetrically (λ = 460 nm) after a 4-h incubation period using a Spectramax microplate spectrophotometer (Molecular Devices; Sunnyvale, CA).

Proteolytic enzyme activity was determined at native substrate concentration within 48 h of field collection by incubating two subsamples (2 g in each subsample) of soil in 50 mL centrifuge tubes for 4 h. Five mL of a trichloroacetic acid (TCA) solution and 0.4 mL of toluene were added to each T_o subsample, which were immediately extracted in 10 mL 0.05 M sodium acetate buffer (pH 5) and filtered through #1 Whatman filters. The addition of toluene prevents microbial uptake of free amino acids and the TCA solution sterilizes the solution (Watanabe and Hayano, 1995). The T_f subsample was incubated in 10 mL 0.05 M sodium acetate buffer and 0.4 mL toluene for 4 h while shaking at 125 rpm. TCA (5 mL) solution was added to each T_f sample after 4 h to halt proteolytic activity and the soil slurries were filtered as stated above. Amino acid concentration in each extract was quantified on a Gemini XS microplate fluorometer (Molecular Devices; Sunnyvale, CA) using the OPAME method (Jones et al., 2002). Native proteolytic enzyme activity (μg N g soil^{-1} hr^{-1}) was then calculated as the

Table 2
Minimum winter soil temperature (°C), maximum soil frost or snow depth (cm), and soil frost or snow depth area under the curve (AUC, cm x days) measured at CCASE. Data collected in winter 2013/14 and 2014/15 were averaged by plot across years prior to statistical analysis. Values are medians with interquartile range (IQR) values. Different superscript letters within a row denote significant differences (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

Response Variable	Reference		Warmed		Warmed + FTC	
	Median	IQR	Median	IQR	Median	IQR
Minimum Temperature***	0.01^{b}	−0.21–0.32	-0.56^{b}	−0.87–−0.34	-4.14^{a}	−4.54–−3.88
Maximum Frost Depth*	12.3^{b}	10.9–13.3	12.6^{b}	11.1–14.2	21.4^{b}	20.7–22.1
Maximum Snow Depth*	63.4^{a}	54.5–72.8	55.8^{a}	52.2–61.2	22.2^{b}	18.2–28.18
Frost AUC*	1100^{b}	1027–1181	1218^{ab}	1165–1262	1455^{a}	1345–1552
Snow AUC***	4057^{a}	3785–4337	3678^{a}	3451–3993	736^{b}	697–754
Freeze-Thaw Cycles	0^{b}		0^{b}		4^{a}	

Table 3
Microbial biomass and activity rates measured at CCASE. Median and interquartile range (IQR) are indicated for each treatment across sampling dates in 2014 and 2015. Different superscript letters within a row denote significant differences (†$P \leq 0.10$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

Response Variable	Reference		Warmed		Warmed + FTC	
	Median	IQR	Median	IQR	Median	IQR
Soil Moisture*	1.4^{a}	0.8–2.2	1.0^{b}	0.7–1.6	0.8^{ab}	0.6–1.2
Extractable Amino Acid-N†	270.2^{a}	147.6–394.2	185.6^{ab}	119.1–298.7	112.9^{b}	64.7–207.4
Extractable Organic C	0.7	0.5–0.8	0.6	0.4–0.7	0.4	0.4–0.6
Dissolved Inorganic N*	9.9^{b}	5.6–19.9	12.9^{b}	8.0–19.0	18.3^{a}	11.4–24.4
Microbial Biomass N	366.9	236.7–511.7	369.2	254.2–524.2	327.4	225.6–442.9
Proteolysis	93.7	59–142	101.8	79–140	75.1	47–127
Acid Phosphatase†	5.3^{a}	3.6–7.8	4.0^{ab}	2.8–6.1	3.4^{b}	2.6–5.2
N-acetylglucosaminidase	0.8	0.5–1.4	0.9	0.6–1.2	0.7	0.4–1
Beta Glucosidase	1.0	0.6–1.7	1.3	0.8–1.8	1.1	0.7–1.7
Cellobiohydrolase	0.4	0.2–0.5	0.3	0.2–0.5	0.3	0.2–0.5
Phenol Oxidase**	0.9^{a}	0.6–1.5	0.8^{a}	0.5–1.2	0.4^{a}	0.3–0.6
Peroxidase***	1.3^{a}	0.9–1.8	1.3^{a}	0.9–1.9	0.9^{b}	0.7–1.2
Net N Mineralization	1.5	0.01–3.5	1.7	0.9–3.8	1.5	0.2–3.7
Microbial Respiration	11.3	8.6–14.5	9.0	7.3–14.2	8.7	6.4–11.9

Units of measurement – Soil Moisture (g H_2O g soil^{-1}), Extractable Amino Acid-N (μg AA-N g soil^{-1}), Extractable Organic C (mg C g soil^{-1}), Dissolved Inorganic N (μg N g soil^{-1}), Microbial Biomass N (μg N g soil^{-1}), AP, NAG, BG, CBH (μmol MUB g soil^{-1} hr^{-1}), phenol oxidase and peroxidase (μmol L-DOPA g soil^{-1} hr^{-1}), Net N mineralization (μg N g soil^{-1} day^{-1}), Microbial Respiration (μg C-CO_2 g soil^{-1} hr^{-1}).

difference in the mass of amino acid N found between the T_f and T_o extracts, divided by the incubation time (4 h).

2.5. Microbial respiration, net N mineralization, and temperature sensitivity

Rates of microbial respiration, net N mineralization, and nitrification were measured in 28-day lab incubations for all samples collected in 2014. Because soils were measured at a constant 20 °C, these are potential as opposed to absolute rates of activity. The samples were incubated in the dark in 500 mL mason jars and extracted with 2 M KCl at the end of the incubation period. We extracted ammonium (NH_4^+) and NO_3^- from subsamples on Day 1 and Day 28 of the incubation and NH_4^+ and NO_3^- were measured colorimetrically using a Versamax microplate spectrophotometer (Doane and Horwath, 2003; Sims et al., 1995). Potential net nitrification (μg N g soil^{-1} day^{-1}) was calculated as the difference in NO_3^- measured on Day 1 and Day 28, correcting for the water content of the soils and dividing by the incubation period. Potential net N mineralization (μg N g soil^{-1} day^{-1}) was calculated as the difference in the sum of NH_4^+ plus NO_3^- measured on Day 1 and Day 28. Microbial respiration was measured four times during the incubation by sampling 5 mL of the jar headspace at hourly intervals over a 3-h period. The gas samples were injected onto an EGM-4 portable infrared gas analyzer (PP-Systems, Amesbury, MA) to determine the rate of microbial respiration (μg C-CO_2 g soil^{-1} hr^{-1}).

To determine the temperature sensitivity of net N mineralization and respiration, we also incubated subsamples collected in 2014 at 4 °C and 20 °C for soils collected following snowmelt in April, and at 12.5 °C, 17.5 °C, 22.5 °C, and 26.5 °C for all other sampling periods. The relationship between soil temperature and net N mineralization or microbial respiration was modeled according to the van't Hoff equation:

$$R_s = \beta \text{ x } \exp(k \text{ x } T) \quad (1)$$

where β is the y-intercept term, k is the exponential decay coefficient, and T is the incubation temperature. The exponential decay coefficient was used to calculate plot- and treatment-specific temperature sensitivity (Q_{10}), which is the factor by which respiration increases for every 10 °C increase in incubation temperature:

$$Q_{10} = \exp(10 \text{ x } k) \quad (2)$$

2.6. Statistical analyses

All statistical analyses were conducted using R v. 3.2.0 (R Development Core Team, 2015). We averaged replicate snow or frost depth measurements and soil or microbial measurements made within each plot to obtain one plot mean for each measurement type on each sampling date. The plot mean for each response variable was used in each statistical analysis. The area under the curve (AUC) of either snow or soil frost depth (y-axis) versus time (x-axis) was determined for each plot using the using the R package 'pracma' (Borchers, 2011), which integrates both the depth and duration of either snow or soil frost as a continuous variable (Durán et al., 2014). Linear mixed-effect models were used to determine the effect of the experimental treatments (i.e. fixed-effect) on minimum winter soil temperature, maximum and minimum snow or soil frost depth, and snow or soil frost depth AUC, as well as soil microbial biomass N, exoenzyme activity, net mineralization, and respiration. Years nested within experimental plots were designated as the random-effects in the models. This analysis was done to

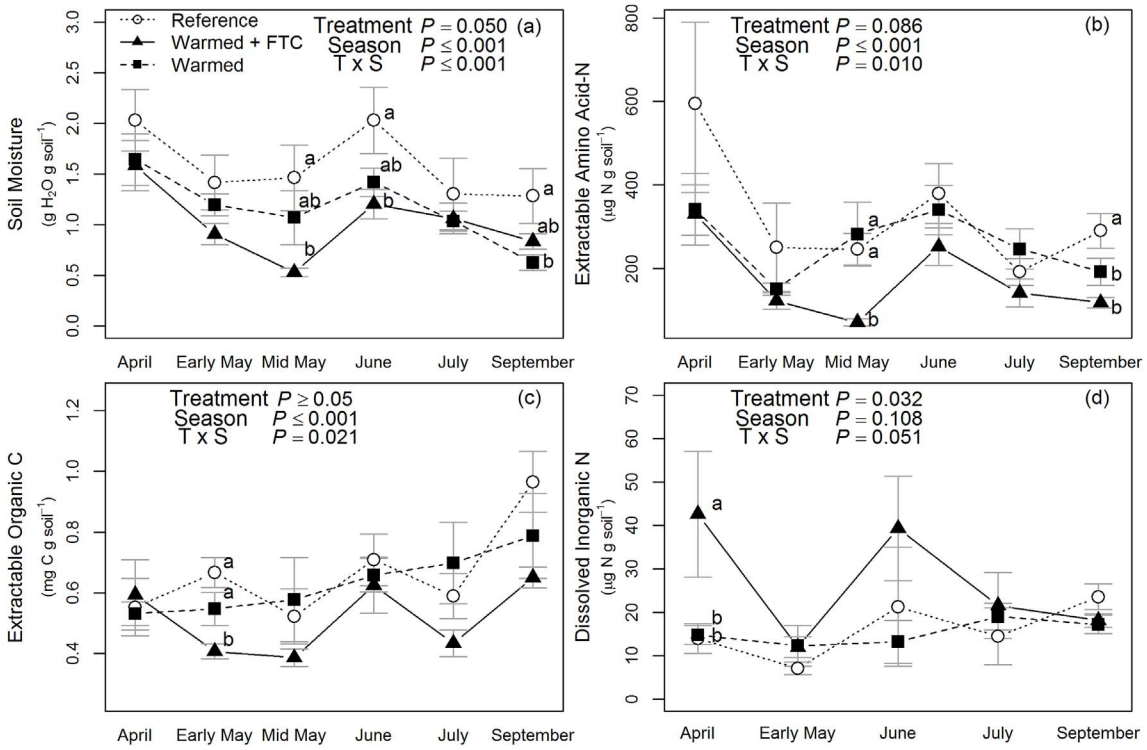

Fig. 1. Soil moisture (a), extractable amino acid-N (b), extractable organic C (c), and dissolved inorganic nitrogen (d) measured at CCASE. Points are means that were averaged across treatments and years (2014 and 2015) ± 1 standard deviation (n = 2). Differences across treatments within a given sampling date were tested using Tukey's HSD and are indicated by different lowercase letters ($P \leq 0.05$).

test for treatment effects both within sampling dates and across all sampling dates. Posthoc pairwise comparisons were made across treatments using the 'multcomp' package (Hothorn et al., 2008). We also tested for correlations between soil moisture and microbial activity or microbial biomass and activity by calculating the marginal R^2 of separate linear mixed-effects models (Nakagawa and Schielzeth, 2013). Soil moisture or microbial biomass was the fixed-effect and microbial activity was the response variable in each statistical model. The random-effects were years nested within plots when testing across sampling dates or plots only when testing within a sampling date. Due to the cost and infrastructure required to implement the experimental treatments, it was not possible to have more than two Warmed and two Warmed + FTC plots, which decreased statistical power and may have limited our ability to detect some differences among treatments. Therefore, P-values ≤ 0.10 were interpreted as a marginally significant treatment effect, which is noted throughout the Results section.

3. Results

We made post-cable installation, pre-treatment measurements of soil bulk density, soil pH, total soil C, total soil N, and soil C:N ratio for soils collected in July 2013 (Table 1). We found no pretreatment differences for any of the measured soil properties prior to initiating the field treatments in winter 2013 (Table 1).

Four winter soil freeze-thaw cycles were induced in the Warmed + FTC treatment in both winter 2013/14 and 2014/15 (Table 2). By contrast, neither the Warmed nor the Reference treatment experienced winter soil freeze-thaw cycles. Because the snowpack was not manipulated and accumulated at ambient rates in the Reference and Warmed treatments, snow depth and duration (i.e. area under the curve = AUC) were five times greater ($P \leq 0.001$) and maximum winter snow depth was three times deeper ($P = 0.019$) in the Reference and Warmed compared to the Warmed + FTC treatment. Similarly, maximum soil frost depth was increased two-fold ($P = 0.014$) and minimum winter soil temperature was approximately 4 °C colder in the Warmed + FTC compared to the Reference or Warmed treatments ($P \leq 0.001$, Table 2). Mean daily soil temperature during the snow-free time of the year was 4.9 ± 0.8 °C higher in the two treatments with soil warming compared to the Reference treatment in both 2014 and 2015.

Soil warming resulted in significant declines in soil moisture content across sampling dates ($P = 0.050$, Table 3). Soil moisture was reduced by approximately 60% in the Warmed + FTC treatment compared to the Reference treatment at the onset of tree leaf-out in mid-May ($P = 0.032$, Fig. 1a). Growing season soil warming combined with winter freeze-thaw cycles also resulted in 30% reductions in soil moisture during June and soil warming alone resulted in a ~50% decline in moisture availability at the onset of plant senescence in September ($P = 0.028$, Fig. 1a).

Extractable amino acid-N was 2.5-fold lower in the Warmed + FTC compared to Reference treatment when averaged across sampling dates ($P = 0.083$, Table 3). The largest differences across treatments occurred in mid-May (Fig. 1b). At that time, the extractable amino acid-N pool was 3x lower in the Warmed + FTC compared to Reference and/or Warmed treatments ($P = 0.018$, Fig. 1b). Similarly, amino acid-N was lower in both warmed treatments compared to the Reference treatment at the onset of plant senescence in September. Like amino acid N pools, extractable organic C was reduced by one-third in the Warmed + FTC compared to Reference or Warmed treatments in early May (Fig. 1c).

In contrast to extractable amino acid-N or organic C pools, dissolved inorganic N (DIN) was two-fold greater in the Warmed + FTC compared to the Reference and Warmed treatments (Table 3). The largest differences across treatments occurred following snowmelt (Fig. 1d), when pools of DIN were about 4-fold greater in the Warmed + FTC treatment compared to the Reference and Warmed treatments ($P = 0.051$, Fig. 1d).

Microbial biomass N declined by 85% following snowmelt in April through tree leaf-out in early-May in soils that had experienced an

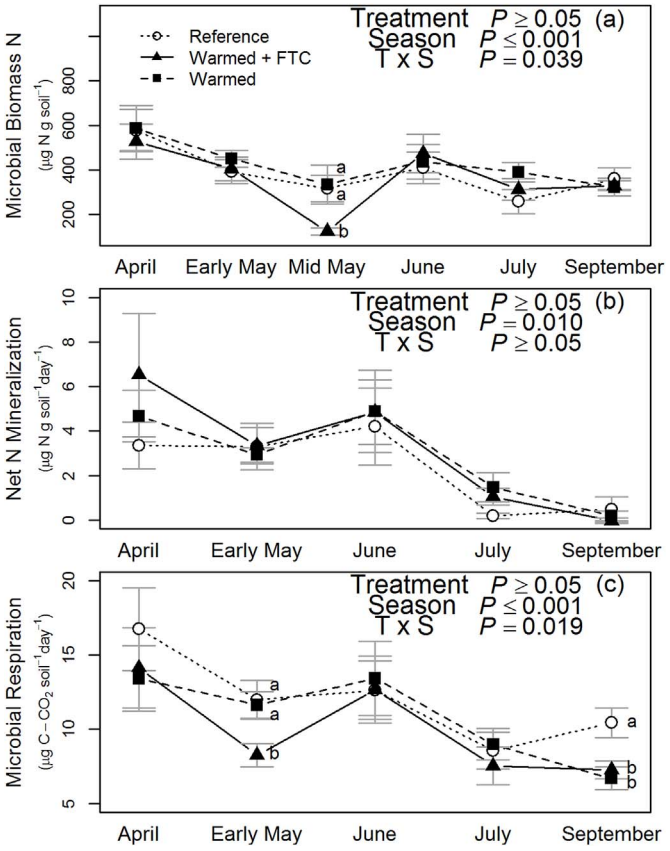

Fig. 2. Seasonal measurements of (a) microbial biomass N, net N mineralization (b), and microbial respiration for soils collected in 2014 (c). Points are means that were averaged across treatments ± 1 standard deviation (n = 2). Differences across treatments within a given sampling date were tested using Tukey's HSD and are indicated by different lowercase letters ($P \leq 0.05$).

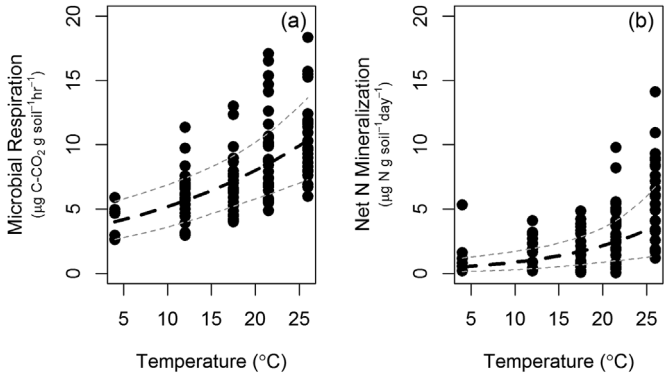

Fig. 3. Temperature response of (a) microbial respiration and (b) net N mineralization measured in lab incubations. Data points are means averaged by plot for soil samples that were collected in 2014. The temperature response of both respiration and net N mineralization did not differ among treatments on any date.

increased frequency of winter soil freeze-thaw cycles (Fig. 2a). Potential net N mineralization did not differ among treatments on any sampling date (Fig. 2b). Potential microbial respiration was 30% lower in the Warmed + FTC treatment compared to the Reference or the Warmed treatments in early May (Fig. 2c). Microbial respiration was correlated positively with soil moisture content at that time ($R^2_{marginal} = 0.65$, $P \leq 0.001$). Likewise, soil warming resulted in lower microbial respiration compared to ambient conditions at the onset of plant senescence in September (Fig. 2c), which was also related to reductions in soil moisture availability in the warmed treatments ($R^2_{marginal} = 0.54$, $P \leq 0.001$). Both potential microbial respiration and net N

Table 4
The temperature sensitivity (Q_{10}) of microbial respiration measured in short-term lab incubations for soils collected in 2014. Different superscript letters within a column indicate significant differences across sampling dates within a treatment ($P \leq 0.05$).

	Reference	Warmed	Warmed + FTC
	Q_{10}	Q_{10}	Q_{10}
April	$2.0^{A} \pm 0.1$	$1.9^{A} \pm 0.2$	1.9 ± 0.2
Mid May	$1.5^{B} \pm 0.2$	$1.5^{B} \pm 0.1$	1.4 ± 0.3
June	$1.5^{B} \pm 0.1$	$1.7^{AB} \pm 0.3$	1.5 ± 0.2
July	$1.6^{B} \pm 0.2$	$1.5^{AB} \pm 0.2$	1.5 ± 0.4
September	$1.8^{AB} \pm 0.5$	$1.8^{AB} \pm 0.4$	1.9 ± 0.5

mineralization rates increased with increasing temperature in lab incubations (Fig. 3). However, there were no differences in the temperature sensitivity of microbial respiration (i.e. Q_{10}) across treatments on any sampling date (Table 4).

Averaged across sampling dates, acid phosphatase, peroxidase, and phenol oxidase activity were all lower in the Warmed + FTC compared to Reference or Warmed treatments (Table 3). For example, phenol oxidase activity was two times lower and peroxidase activity was reduced by 20% in the Warmed + FTC treatment compared to the Reference or Warmed treatments (Table 3). Soil moisture availability was related positively to acid phosphatase ($R^2_{marginal} = 0.57$, $P \leq 0.001$), phenol oxidase ($R^2_{marginal} = 0.25$, $P \leq 0.001$), and peroxidase activity ($R^2_{marginal} = 0.08$, $P \leq 0.001$) across sampling dates.

Soil warming alone had no stimulatory effect on proteolytic or oxidative enzyme activity compared to the Reference treatment (Table 3, Fig. 4b,c,d). Winter soil freeze-thaw cycles reduced proteolytic enzyme activity by 75% in mid-May (Fig. 4b); and reductions in proteolytic activity were significantly correlated with reductions in microbial biomass N at that time ($R^2_{marginal} = 0.86$, $P \leq 0.001$). Likewise, phenol oxidase activity was 30% lower compared to the Reference or Warmed treatments at the onset of tree leaf-out in mid-May (Fig. 4b,c,d). Whereas differences in proteolytic enzyme activity across treatments were observed in mid-May only (Fig. 4b), winter soil freeze-thaw cycles resulted in persistent declines in oxidative enzyme activity that were observed on most sampling dates (Fig. 4c and d).

4. Discussion

Rising air temperatures have reduced the depth and duration of winter snow cover in many ecosystems that experience a seasonal snowpack (Burakowski et al., 2008; Henry, 2008; Kreyling and Henry, 2011). Yet, the impact of less winter snow and an increased frequency of soil freeze-thaw cycles combined with growing season soil warming on soil C and N cycling are largely unknown. To address this knowledge gap we implemented an experiment in a northern forest ecosystem with field plots that are either warmed during the snow-free times of the year only or experience growing season soil warming in combination with an increased frequency of soil freeze-thaw cycles in winter (Templer et al., 2017). We induced four winter soil freeze-thaw cycles in each year of the study, which is consistent with projected increases in the frequency of winter soil freeze-thaw for forests in the northeastern U.S. region (Campbell et al., 2010).

4.1. Soil warming does not compensate for reductions due to winter soil freeze-thaw

We hypothesized that soil warming would lead to elevated microbial biomass and rates of microbial activity across the snow-free times of the year. Although potential rates of microbial respiration and net N mineralization increased exponentially with incubation temperature in the lab (Fig. 3), we did not observe any stimulatory effect from the soil warming treatment relative to ambient conditions on microbial biomass

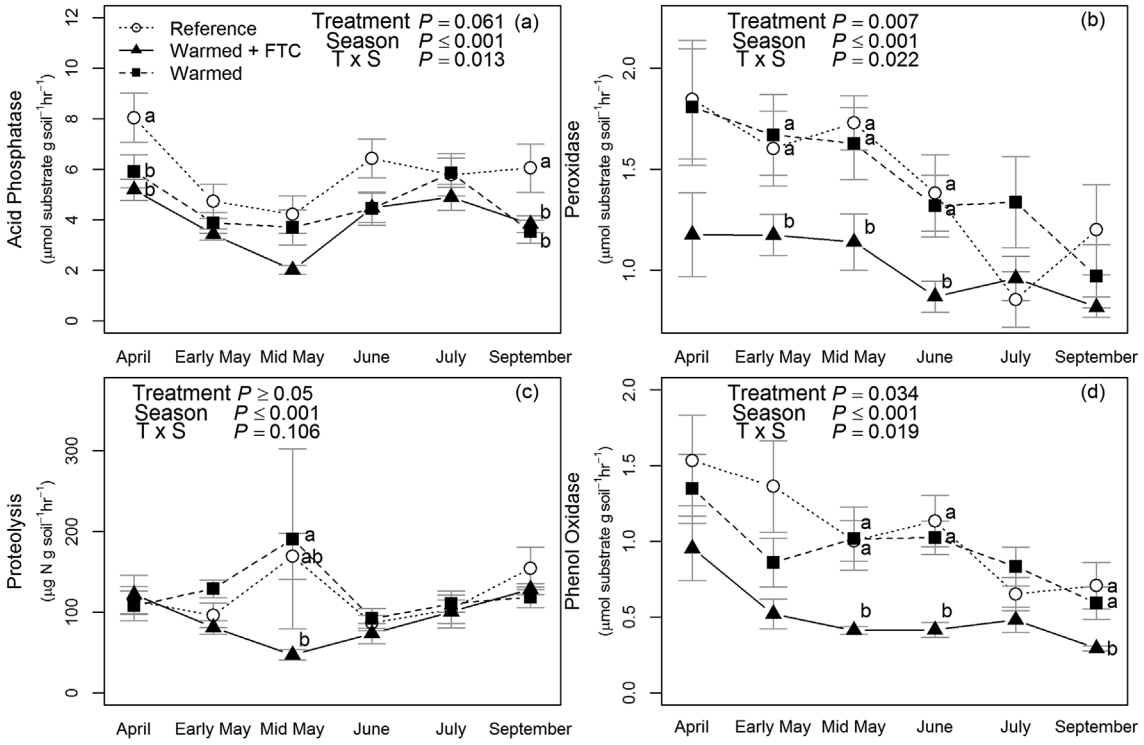

Fig. 4. Seasonal measurements of hydrolytic and oxidative soil extracellular enzymes. Points are means that were averaged across treatments and years (2014 and 2015) ± 1 standard deviation (n = 2). Differences across treatments within a given sampling date were tested using Tukey's HSD and are indicated by different lowercase letters ($P \leq 0.05$).

or activity at any sampling time. These results stand in contrast to significant increases in microbial respiration and soil net N mineralization in response to +5 °C soil warming at nearby Harvard Forest, MA (Melillo et al., 2002), but are similar to other studies that have also observed either no effect, or in many cases reductions, in potential exoenzyme activity, net N mineralization, and microbial respiration associated with soil warming that can often be attributed to declines in soil moisture availability and substrate supply which thereby limits microbial activity (Abramoff et al., 2017; Allison and Treseder, 2008; Henry, 2012; Jing et al., 2014; Kardol et al., 2010; Schindlbacher et al., 2015; Steinweg et al., 2012; Weedon et al., 2014).

In support of Hypothesis 2, we found that the combination of growing season warming and winter soil freeze-thaw cycles resulted in significant reductions in soil pools of extractable amino acid-N and dissolved organic C, proteolytic and oxidative enzyme activity, and microbial respiration following snowmelt through tree leaf-out in spring. These declines in microbial activity were concurrent with an 85% decline in microbial biomass N. The lack of a winter soil freeze-thaw cycle only treatment in our experimental design precludes our ability to quantify the effect of soil freeze-thaw cycles in isolation of warming and/or the effects of multiple shorter duration freeze-thaw cycles versus a single frost event of long duration (as could be expected from snow-removal, see discussion below). Still, we infer that the reductions in soil nutrient pools, microbial biomass, and enzyme activity are more strongly driven by winter soil freeze-thaw cycles, rather than the combination of soil warming and winter soil freeze-thaw cycles, because there were no differences in soil microbial activity or soil nutrient pools sizes among the Warmed and Reference treatments during any time period.

4.2. Freeze-thaw reduces microbial biomass and activity in spring

Reductions in microbial biomass and activity during spring that are induced by winter soil freeze-thaw cycles have important implications for the northern forest region because rising winter air temperatures have reduced the duration of winter snow cover and spring snowmelt has occurred approximately four days per decade earlier over the last 50 years in this region (Hamburg et al., 2013). Groffman et al. (2012) hypothesized that earlier snowmelt, due to declining snowpacks and warmer spring air temperatures, might decouple microbial-derived nutrient supply from plant nutrient demand by inducing an earlier onset of microbial-relative to plant activity in spring. The results from our study indicate that an increased frequency of winter soil freeze-thaw cycles will result in substantial reductions in springtime microbial biomass and activity. Therefore, an increased frequency of winter soil freeze-thaw cycles is likely to partially mitigate some effects of asynchrony between microbial and plant phenology (*sensu* Groffman et al., 2012; Abramoff and Finzi, 2016) due to a lengthening vernal window caused by earlier snowmelt (Contosta et al., 2016).

4.3. Effects of single versus multiple freeze-thaw cycles

We expected that reductions in microbial biomass or activity due to an increased frequency of winter soil freeze-thaw cycles would be limited to several weeks following spring snowmelt and not persist later into the year because our past snow removal experiments documented only short-term, transient effects of winter soil freezing on microbial biomass and activity (Sorensen et al., 2016). However, in this study an increased frequency of winter soil freeze-thaw cycles resulted in two-fold reductions in phenol oxidase and a 20% decline in peroxidase activity that persisted across the growing season. Notably in the Warmed treatment, there were no reductions in oxidative enzyme activity. The persistent decline in oxidative activity due to winter soil freeze-thaw cycles is an important finding because the activity of these oxidative enzymes mediate the breakdown of recalcitrant soil organic C and thereby influence soil C storage as well as N mineralization rates (Bodeker et al., 2014; Sinsabaugh, 2010). Because the snow removal treatment used in many studies often produces a single soil freeze event of long duration (Hardy et al., 2001; Campbell et al., 2014), the patterns shown in this study also suggest that multiple winter soil freeze-thaw cycles are a more significant soil disturbance for soil oxidative enzyme activity compared to a single soil freezing event of longer

duration. The distinction in the response to a single freeze event of long-duration freeze versus multiple soil freeze-thaw events is important because the frequency of soil freeze-thaw cycles is expected to increase and the duration of winter soil frost is expected to decline over the next 100 years in the northern forest region (Hayhoe et al., 2007; Campbell et al., 2010; Brown and Degaetano, 2011).

5. Conclusion

Air temperatures are rising during both winter and the snow-free months of the year, which will significantly alter soil C and N cycling in northern forest ecosystems. The results of this study indicate that winter soil freeze-thaw cycles will diminish standing pools of organic N and C, as well as decrease the activity of some exoenzymes such as proteases and acid phosphatase. Moreover, persistent declines in phenol oxidase and peroxidase activity do not appear to be compensated for by warmer soil temperatures occurring during snow-free periods of the year. If the patterns we observed at the CCASE experiment hold true across other temperate forest locations which experience a seasonal snowpack, the results from this study indicate that winter soil freeze-thaw cycles will partially mitigate positive effects of soil warming on soil microbial pools and process rates during the coming century.

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References

Aanderud, Z.T., Jones, S.E., Schoolmaster Jr., D.R., Fierer, N., Lennon, J.T., 2013. Sensitivity of soil respiration and microbial communities to altered snowfall. Soil Biology & Biochemistry 57, 217–227.

Abramoff, R.Z., Davidson, E.A., Finzi, A.C., 2017. A parsimonious modular approach to building a mechanistic belowground carbon and nitrogen model. Journal of Geophysical Research: Biogeosciences. http://dx.doi.org/10.1002/2017JG003796.

Abramoff, R.Z., Finzi, A.C., 2016. Seasonality and partitioning of root allocation to rhizosphere soils in a midlatitude forest. Ecosphere 7 e01547-n/a.

Aerts, R., Cornelissen, J.H.C., Dorrepaal, E., van Logtestijn, R.S.P., Callaghan, T.V., 2004. Effects of experimentally imposed climate scenarios on flowering phenology and flower production of subarctic bog species. Global Change Biology 9, 1599–1609.

Allison, S.D., Treseder, K.K., 2008. Warming and drying suppress microbial activity and carbon cycling in boreal forest soils. Global Change Biology 14, 2898–2909.

Aronson, E.L., McNulty, S.G., 2009. Appropriate experimental ecosystem warming methods by ecosystem, objective, and practicality. Agricultural and Forest Meteorology 149, 1791–1799.

Bai, E., Li, S.L., Xu, W.H., Li, W., Dai, W.W., Jiang, P., 2013. A meta-analysis of experimental warming effects on terrestrial nitrogen pools and dynamics. New Phytologist 199, 441–451.

Bailey, A., Hornbeck, J., Campbell, J., Eagar, C., 2003. Hydrometeorological database for Hubbard Brook experimental forest: 1955-200. In: General Technical Report. US Department of Agriculture, Northeastern Reseach Station, Newton Square, PA.

Bartlett, R.J., Ross, D.S., 1988. Colorimetric determination of oxidizable carbon in acid soil solutions. Soil Science Society of America Journal 52, 1191–1192.

Bodeker, I.T.M., Clemmensen, K.E., de Boer, W., Martin, F., Olson, A., Lindahl, B.D., 2014. Ectomycorrhizal Cortinarius species participate in enzymatic oxidation of humus in northern forest ecosystems. New Phytologist 203, 245–256.

Bohlen, P.J., Groffman, P.M., Driscoll, C.T., Fahey, T.J., Siccama, T.G., 2001. Plant-soil-microbial interactions in a northern hardwood forest. Ecology 82, 965–978.

Borchers, H.W., 2011. pracma: Practical Numerical Math Functions. R package version 0.3-0.

Boutin, R., Robitaille, G., 1995. Increased soil nitrate losses under mature sugar maple trees affected by experimentally induced deep frost. Canadian Journal of Forest Research 25, 588–602.

Brookes, P.C., Landman, A., Pruden, G., Jenkinson, D.S., 1985. Chloroform Fumigation and the release of of soil nitroge - a rapid direct extraction method to measure microbial biomass nitrogen in soil. Soil Biology & Biochemistry 17, 837–842.

Brooks, P.D., Williams, M.W., 1999. Snowpack controls on nitrogen cycling and export in seasonally snow-covered catchments. Hydrological Processes 13, 2177–2190.

Brown, P.J., DeGaetano, A.T., 2011. A paradox of cooling winter soil surface temperatures in a warming northeastern United States. Agricultural and Forest Meteorology 151, 947–956.

Brzostek, E.R., Finzi, A.C., 2011. Substrate supply, fine roots, and temperature control proteolytic enzyme activity in temperate forest soils. Ecology 92, 892–902.

Burakowski, E.A., Wake, C.P., Braswell, B., Brown, D.P., 2008. Trends in wintertime climate in the northeastern United States: 1965-2005. Journal of Geophysical Research-atmospheres 113.

Cabrera, M.L., Beare, M.H., 1993. Alkaline persulfate oxidation for determining total nitrogen in microbial biomass extracts. Soil Science Society of America Journal 57, 1007–1012.

Campbell, J.L., Ollinger, S.V., Flerchinger, G.N., Wicklein, H., Hayhoe, K., Bailey, A.S., 2010. Past and projected future changes in snowpack and soil frost at the Hubbard Brook Experimental Forest, New Hampshire, USA. Hydrological Processes 24, 2465–2480.

Campbell, J.L., Socci, A.M., Templer, P.H., 2014. Increased nitrogen leaching following soil freezing is due to decreased root uptake in a northern hardwood forest. Global Change Biology 20, 2662–2673.

Contosta, A.R., Adolph, A., Burchsted, D., Burakowski, E., Green, M., Guerra, D., Albert, M., Dibb, J., Martin, M., McDowell, W.H., Routhier, M., Wake, C., Whitaker, R., Wollheim, W., 2016. A longer vernal window: the role of winter coldness and snowpack in driving spring transitions and lags. Global Change Biology 23, 1610–1625.

Cox, P.M., Betts, R.A., Jones, C.D., Spall, S.A., Totterdell, I.J., 2000. Acceleration of global warming due to carbon-cycle feedbacks in a coupled climate model. Nature 408, 184–187.

Davidson, E.A., Janssens, I.A., 2006. Temperature sensitivity of soil carbon decomposition and feedbacks to climate change. Nature 440, 165–173.

De Frenne, P., 2015. Innovative empirical approaches for inferring climate-warming impacts on plants in remote areas. New Phytologist 205, 1015–1021.

Deluca, T.H., Keeney, D.R., McCarty, G.W., 1992. Effect of freeze-thaw events on mineralization of soil-nitrogen. Biology and Fertility of Soils 14, 116–120.

Doane, T.A., Horwath, W.R., 2003. Spectrophotometric determination of nitrate with a single reagent. Analytical Letters 36, 2713–2722.

Drake, J.E., Giasson, M.A., Spiller, K.J., Finzi, A.C., 2013. Seasonal plasticity in the temperature sensitivity of microbial activity in three temperate forest soils. Ecosphere 4, 21.

Durán, J., Morse, J.L., Groffman, P.M., Campbell, J.L., Christenson, L.M., Driscoll, C.T., Fahey, T.J., Fisk, M.C., Mitchell, M.J., Templer, P.H., 2014. Winter climate change affects growing-season soil microbial biomass and activity in northern hardwood forests. Global Change Biology 20, 3568–3577.

Fahey, T.J., Lang, G.E., 1975. Concrete frost along an elevational gradient in New Hampshire. Canadian Journal of Forest Research 5, 700–705.

Fahey, T.J., Tierney, G.L., Fitzhugh, R.D., Wilson, G.F., Siccama, T.G., 2005. Soil respiration and soil carbon balance in a northern hardwood forest ecosystem. Canadian Journal of Forest Research-revue Canadienne De Recherche Forestiere 35, 244–253.

Fitzhugh, R.D., Driscoll, C.T., Groffman, P.M., Tierney, G.L., Fahey, T.J., Hardy, J.P., 2001. Effects of soil freezing disturbance on soil solution nitrogen, phosphorus, and carbon chemistry in a northern hardwood ecosystem. Biogeochemistry 56, 215–238.

Friedlingstein, P., Cox, P., Betts, R., Bopp, L., Von Bloh, W., Brovkin, V., Cadule, P., Doney, S., Eby, M., Fung, I., Bala, G., John, J., Jones, C., Joos, F., Kato, T., Kawamiya, M., Knorr, W., Lindsay, K., Matthews, H.D., Raddatz, T., Rayner, P., Reick, C., Roeckner, E., Schnitzler, K.G., Schnur, R., Strassmann, K., Weaver, A.J., Yoshikawa, C., Zeng, N., 2006. Climate-carbon cycle feedback analysis: results from the (CMIP)-M-4 model intercomparison. Journal of Climate 19, 3337–3353.

Giasson, M.A., Averill, C., Finzi, A.C., 2014. Correction factors for dissolved organic carbon extracted from soil, measured using the Mn(III)-pyrophosphate colorimetric method adapted for a microplate reader. Soil Biology & Biochemistry 78, 284–287.

Giasson, M.A., Ellison, A.M., Bowden, R.D., Crill, P.M., Davidson, E.A., Drake, J.E., Frey, S.D., Hadley, J.L., Lavine, M., Melillo, J.M., Munger, J.W., Nadelhoffer, K.J., Nicoll, L., Ollinger, S.V., Savage, K.E., Steudler, P.A., Tang, J., Varner, R.K., Wofsy, S.C., Foster, D.R., Finzi, A.C., 2013. Soil respiration in a northeastern US temperate forest: a 22-year synthesis. Ecosphere 4.

Giblin, A.E., Nadelhoffer, K.J., Shaver, G.R., Laundre, J.A., McKerrow, A.J., 1991. Biogeochemical diversity along a riverside toposequence in arctic Alaska. Ecological Monographs 61, 415–435.

Groffman, P.M., Altabet, M.A., Bohlke, J.K., Butterbach-Bahl, K., David, M.B., Firestone, M.K., Giblin, A.E., Kana, T.M., Nielsen, L.P., Voytek, M.A., 2006. Methods for measuring denitrification: diverse approaches to a difficult problem. Ecological

Applications 16, 2091–2122.
Groffman, P.M., Driscoll, C.T., Fahey, T.J., Hardy, J.P., Fitzhugh, R.D., Tierney, G.L., 2001. Colder soils in a warmer world: a snow manipulation study in a northern hardwood forest ecosystem. Biogeochemistry 56, 135–150.
Groffman, P.M., Rustad, L.E., Templer, P.H., Campbell, J.L., Christenson, L.M., Lany, N.K., Socci, A.M., Vadeboncoeur, M.A., Schaberg, P.G., Wilson, G.F., Driscoll, C.T., Fahey, T.J., Fisk, M.C., Goodale, C.L., Green, M.B., Hamburg, S.P., Johnson, C.E., Mitchell, M.J., Morse, J.L., Pardo, L.H., Rodenhouse, N.L., 2012. Long-term integrated studies show complex and surprising effects of climate change in the Northern Hardwood forest. Bioscience 62, 1056–1066.
Hamburg, S.P., Vadeboncoeur, M.A., Richardson, A.D., Bailey, A.S., 2013. Climate change at the ecosystem scale: a 50-year record in New Hampshire. Climatic Change 116, 457–477.
Hardy, J.P., Groffman, P.M., Fitzhugh, R.D., Henry, K.S., Welman, A.T., Demers, J.D., Fahey, T.J., Driscoll, C.T., Tierney, G.L., Nolan, S., 2001. Snow depth manipulation and its influence on soil frost and water dynamics in a northern hardwood forest. Biogeochemistry 56, 151–174.
Hayhoe, K., Wake, C.P., Huntington, T.G., Luo, L.F., Schwartz, M.D., Sheffield, J., Wood, E., Anderson, B., Bradbury, J., DeGaetano, A., Troy, T.J., Wolfe, D., 2007. Past and future changes in climate and hydrological indicators in the US Northeast. Climate Dynamics 28, 381–407.
Henry, H.A.L., 2008. Climate change and soil freezing dynamics: historical trends and projected changes. Climatic Change 87, 421–434.
Henry, H.A.L., 2012. Soil extracellular enzyme dynamics in a changing climate. Soil Biology & Biochemistry 47, 53–59.
Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biometrical Journal 50, 346–363.
Jing, X., Wang, Y.H., Chung, H.G., Mi, Z.R., Wang, S.P., Zeng, H., He, J.S., 2014. No temperature acclimation of soil extracellular enzymes to experimental warming in an alpine grassland ecosystem on the Tibetan Plateau. Biogeochemistry 117, 39–54.
Jones, D.L., Owen, A.G., Farrar, J.F., 2002. Simple method to enable the high resolution determination of total free amino acids in soil solutions and soil extracts. Soil Biology & Biochemistry 34, 1893–1902.
Kardol, P., Cregger, M.A., Campany, C.E., Classen, A.T., 2010. Soil ecosystem functioning under climate change: plant species and community effects. Ecology 91, 767–781.
Kreyling, J., Henry, H.A.L., 2011. Vanishing winters in Germany: soil frost dynamics and snow cover trends, and ecological implications. Climate Research 46, 269–276.
Kuhnert, R., Oberkofler, I., Peintner, U., 2012. Fungal growth and biomass development is boosted by plants in snow-covered soil. Microbial Ecology 64, 79–90.
Lu, M., Zhou, X.H., Yang, Q., Li, H., Luo, Y.Q., Fang, C.M., Chen, J.K., Yang, X., Li, B., 2013. Responses of ecosystem carbon cycle to experimental warming: a meta-analysis. Ecology 94, 726–738.
Matzner, E., Borken, W., 2008. Do freeze-thaw events enhance C and N losses from soils of different ecosystems? A review. European Journal of Soil Science 59, 274–284.
Melillo, J.M., Butler, S., Johnson, J., Mohan, J., Steudler, P., Lux, H., Burrows, E., Bowles, F., Smith, R., Scott, L., Vario, C., Hill, T., Burton, A., Zhou, Y.M., Tang, J., 2011. Soil warming, carbon-nitrogen interactions, and forest carbon budgets. Proceedings of the National Academy of Sciences of the United States of America 108, 9508–9512.
Melillo, J.M., Steudler, P.A., Aber, J.D., Newkirk, K., Lux, H., Bowles, F.P., Catricala, C., Magill, A., Ahrens, T., Morrisseau, S., 2002. Soil warming and carbon-cycle feedbacks to the climate system. Science 298, 2173–2176.
Nakagawa, S., Schielzeth, H., 2013. A general and simple method for obtaining R2 from generalized linear mixed-effects models. Methods in Ecology and Evolution 4, 133–142.
R Development Core Team, 2015. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Reinmann, A.B., Templer, P.H., Campbell, J.L., 2012. Severe soil frost reduces losses of carbon and nitrogen from the forest floor during simulated snowmelt: a laboratory experiment. Soil Biology & Biochemistry 44, 65–74.
Rickard, W., Brown, J., 1972. Performance of a frost-tube for determination of soil freezing and thawing depths. Soil Science 113, 149–154.
Rustad, L.E., Campbell, J.L., Marion, G.M., Norby, R.J., Mitchell, M.J., Hartley, A.E., Cornelissen, J.H.C., Gurevitch, J., Gcte, N., 2001. A meta-analysis of the response of soil respiration, net nitrogen mineralization, and aboveground plant growth to experimental ecosystem warming. Oecologia 126, 543–562.
Sanders-DeMott, R., Templer, P.H., 2017. What about winter? Integrating the missing season into climate change experiments in seasonally snow covered ecosystems. Methods in Ecology and Evolution. http://dx.doi.org/10.1111/2041-1210X.12780.
Schindlbacher, A., Schnecker, J., Takriti, M., Borken, W., Wanek, W., 2015. Microbial physiology and soil CO2 efflux after 9 years of soil warming in a temperate forest - no indications for thermal adaptations. Global Change Biology 21, 4265–4277.
Sims, G.K., Ellsworth, T.R., Mulvaney, R.L., 1995. Microscale determination of inorganic nitrogen in water and soil extracts. Communications in Soil Science and Plant Analysis 26, 303–316.
Sinsabaugh, R.L., 2010. Phenol oxidase, peroxidase and organic matter dynamics of soil. Soil Biology & Biochemistry 42, 391–404.
Skogland, T., Lomeland, S., Goksoyr, J., 1988. Respiratory burst after freezing and thawing of soil - experiments with soil bacteria. Soil Biology & Biochemistry 20, 851–856.
Sorensen, P.O., Templer, P.H., Finzi, A.C., 2016. Contrasting effects of winter snowpack and soil frost on growing season microbial biomass and enzyme activity in two mixed-hardwood forests. Biogeochemistry 128, 141–154.
Steinweg, J.M., Dukes, J.S., Wallenstein, M.D., 2012. Modeling the effects of temperature and moisture on soil enzyme activity: linking laboratory assays to continuous field data. Soil Biology & Biochemistry 55, 85–92.
Templer, P.H., Reinmann, A.B., Sanders-DeMott, R., Sorensen, P.O., Juice, S.M., Bowles, F., Sofen, L.E., Harrison, J.L., Halm, I., Rustad, L., Martin, M.E., Grant, N., 2017. Climate Change across Seasons Experiment (CCASE): a new method for simulating future climate in seasonally snow-covered ecosystems. PLoS One 12 e0171928.
Ueda, M.U., Muller, O., Nakamura, M., Nakaji, T., Hiura, T., 2013. Soil warming decreases inorganic and dissolved organic nitrogen pools by preventing the soil from freezing in a cool temperate forest. Soil Biology & Biochemistry 61, 105–108.
Watanabe, K., Hayano, K., 1995. Seasonal-variation of soil protease activities and their relation to proteolytic bacteria and *Bacillus spp.* in paddy field soil. Soil Biology & Biochemistry 27, 197–203.
Weedon, J.T., Aerts, R., Kowalchuk, G.A., van Bodegom, P.M., 2014. No effects of experimental warming but contrasting seasonal patterns for soil peptidase and glycosidase enzymes in a sub-arctic peat bog. Biogeochemistry 117, 55–66.
Zhang, T.J., 2005. Influence of the seasonal snow cover on the ground thermal regime: an overview. Reviews of Geophysics 43.